REMARKS/ARGUMENTS

Claims 36-49 are currently pending. Claims 36 and 46 are amended as set forth in detail below. No new matter is added. Applicants reserve the right to prosecute claims of original scope in a related, co-pending application. Examination and reconsideration of all pending claims is respectfully requested.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 36-49 remain rejected under 35 U.S.C. § 112, first paragraph, as allegedly not enabled by the specification. In maintaining the present rejection over Applicants' previous arguments, the Examiner relies on new grounds of rejection. These new grounds of rejection are overcome in part and traversed in part as set forth below.

First, while not agreeing with the Examiner's rejection or reasons for rejection, but in order to expedite prosecution of the instant application, Applicants have amended independent claims 36 and 46 to recite that the compound "is an analog of a core 2 GLcNAc transferase substrate." Support for this amendment is found in the application as filed at, for example, page 20, lines 26-28. Support is also found at, *e.g.*, page 24, lines 13-15 (stating that competitive inhibitors typically "resemble the substrate or the product(s) and bind the active site of the enzyme, thus blocking the substrate from binding the active site." Applicants note that, in view of this amendment, any bases for rejection pertaining to the use of antisense molecules[1] are obviated.

Further, before addressing the Examiner's remarks in detail, Applicants note that the Examiner has acknowledged that, if C2GlcNAc transferase protein were sufficiently inhibited *in vivo*, "an inflammatory response would likely be inhibited in a mammal."[2] Thus, the Examiner accepts the predictability of the phenotype achieved should C2GlcNAc be inhibited. According to the Examiner, however, the issue to be addressed is an alleged unpredictability in

[1] *See* Office Action dated August 24, 2005, at pp. 8 & 9.
[2] *Id.* at p. 10.

achieving *in vivo* enzyme inhibition with an inhibitory compound. The Examiner states the following:

> The unpredictability lies not in predicting the phenotype - should the transferase be inhibited - the unpredictability lies in determining whether effective inhibitor concentrations can be obtained in vivo to inhibit this enzyme, following administration of the drug candidate.... [T]he question to be answered is whether any proposed inhibitors can provide treatment effects upon administration in vivo.[3]

In view of the present amendments to the claims, and in view of the Examiner's remarks noted above, Applicants believe that the sole issue to be addressed in this case is whether the skilled artisan would reasonably accept that effective *in vivo* inhibition of C2GlcNAc transferase could be readily achieved using substrate analogs of C2GlcNAc transferase. For the reasons set forth further herein, it is submitted that the skilled artisan, reading the specification, would have regarded effective *in vivo* inhibition of C2GlcNAc transferase using a substrate analog as reasonably predictable.

In the context of the issue set forth above, Applicants will address in turn the Examiner's specific remarks regarding each of the following factors raised in the Office Action:

(1) the state of the prior art and the predictability or unpredictability of the art;

(2) the amount of direction or guidance presented in the specification and the presence or absence of working examples; and

(3) the breadth of claims and the quantity of experimentation.[4]

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[3] *Id.*
[4] *See* Office Action dated August 24, 2005, at pp. 4-7.

The state of the prior art and the predictability of the art

With regard to the state of the prior art and predictability or unpredictability of the art, the Examiner sets forth the following proposition:

> ... the delivery to a target cell in vitro or in vivo of candidate inhibitory molecules, including polypeptides and/or small molecules, is energy dependent and may require the presence of specific proteins that serve as receptor and or channels to provide the intracellular concentrations of small molecule inhibitors required for inhibition.[5]

As alleged support for the above, the Examiner cites to three references: Derossi *et al.*[6], Pooga, *et al.*[7]; and Elliot *et al.*[8]

Applicants submit that this aspect of the rejection is obviated in view of the present claim amendments reciting substrate analogs of C2GlcNAc transferase. Each of the three cited references pertain only to penetration of relatively large polypeptides through cell membranes. In particular, Derossi is directed to the cellular membrane translocation of the 60-amino-acid-long antennapedia homeodomain; Pooga discusses cell penetration by the 27-amino-acid-long peptide transportan; and Elliot shows intercellular transport by the 38 kDa HSV-1 structural protein VP22. None of these references are pertinent to the ability of small molecules, such as substrate analogs of glycosyltransferases, to pass through cellular membranes.

The Examiner further cites to various references pertaining to antisense gene therapy as allegedly showing unpredictability in delivery of oligonucleotides *in vivo*.[9] As previously noted, this aspect of the rejection, which is based on an alleged unpredictability of gene therapy, is also obviated by the present amendments reciting substrate analog inhibitors.

In view of the above, Applicants believe that the submission of rebuttal evidence is not required with respect to this aspect of the rejection. In order to expedite prosecution of this

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[5] *Id.* at p. 4.
[6] *J. Biol. Chem.* 269:10,444-10,450, 1994.
[7] *FASEB J.* 12:67-77, 1998.
[8] *Cell* 88:223-233, 1997.
[9] *See* Office Action dated August 24, 2005, at pp. 8 & 9.

application, however, Applicants have attached hereto Exhibit C[10] (also referred to herein as "Camenisch *et al.*") as evidence to show that, as of the effective filing date, it was well-known that small molecules can predictably penetrate cellular membranes, including biological membrane barriers such as the epithelial layer of the intestine, by passive diffusion.[11] In particular, the ability of a particular small molecule to penetrate a cellular membrane by passive diffusion could be readily predicted based simply on the compound's lipophilicity and molecular size.[12] Moreover, as stated by Camenisch *et al.*, "[p]assive diffusion is the most significant transport mechanism of the majority of drugs."[13] Thus, the skilled artisan would view passive diffusion across cellular membranes as indicative of a compound's ability to be absorbed and to reach its site of action.

In light of this state of the art, and while Applicants accept that active transport mechanisms can contribute to translocation of certain small molecules, it is respectfully submitted that it would have been a routine matter for the skilled artisan to predict whether a particular substrate analog of C2GlcNAc transferase could cross a cellular membrane by passive diffusion using a computational method such as described in Camenisch *et al.*, that such predictions could be confirmed by routine, *in vitro* cell-based assays, and that such methods are further predictive of a compounds ability to be delivered *in vivo*.

Accordingly, in view of the above, the skilled artisan would regard *in vivo* delivery of a small molecule C2GlcNAc transferase inhibitor, including substrate analogs as presently recited in the claims, as reasonably predictable. Concomitantly, because the Examiner has acknowledged that an inflammatory response would likely be inhibited in a mammal, should C2GlcNAc transferase be effectively inhibited, it is further submitted that achieving the claimed *in vivo* effects with such substrate analog inhibitors is also reasonably predictable in light of the specification's teachings.

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[10] Camenisch *et al.*, *Eur J Pharm Sci.* 6:317-24, 1998.
[11] *See id.* at, *e.g.*, p. 313, cols. 1 & 2.
[12] *See id.* at, e.g., p. 313 (Abstract).
[13] *Id.* at p. 313, col. 2, first full para.

The amount of guidance in the specification

With respect to the guidance provided in the specification, including the examples, the Examiner states that there is "a lack of guidance in the specification" and "known unpredictability" with respect to inhibition of glycosyltransferases in vitro or in vivo comprising the administration of sugar substrate analogs. The Examiner contends that the "successful generation of null mice is not representative of the ability to successfully target and inhibit C2 GlcNAc transferase in vivo comprising the administration of inhibitors"; and, further, that "in vitro results cannot be extrapolated to in vivo inhibition and subsequent treatment effects." [14] It is the Examiner's view that *in vivo* results require undue experimentation, and "cannot be generalized from a test tube (or cell culture) to an organism."[15] Applicants address each of these contentions below.

First, Applicants disagree with the Examiner's reliance on a blanket assertion that *in vivo* results require undue experimentation, and "cannot be generalized from a test tube (or cell culture) to an organism." Applicants have indeed demonstrated *in vivo* results in the knockout mouse model, and the Examiner has acknowledged that this demonstration is correlative of an effect that would likely be achieved by inhibition of C2 GlcNAc transferase *in vivo*. Moreover, the Examiner has not cited to any statutory or judicial authority to support the proposition that *in vivo* results require undue experimentation *per se*. For reasons set forth further herein, in view of the knowledge in the art and the guidance provided in the specification, it is submitted that any experimentation in carrying out the method as claimed would not be considered undue by the skilled artisan.

As to the guidance provided in the instant application, according to the MPEP, "[h]ow a teaching is set forth, by specific example or broad terminology, is not important."[16] Further, the specification need not disclose, and preferably omits, what is well-known in the art and already available to the public.[17] Here, it is submitted that the specification provides more than sufficient guidance in view of the level of knowledge and skill in the art, including, *e.g.*, the

[14] Office Action dated August 24, 2005, at p. 6.
[15] Office Action dated August 24, 2005, at p. 6.
[16] MPEP § 2164.08 (citing *In re Marzocchi*, 439 F.2d 220, 223-4, 169 USPQ 367, 370 (CCPA 1971)).

state of the art with respect to substrate analog inhibitors of glycosyltransferases and their previous *in vivo* use, as well as predictability of biological membrane penetrability of small molecules.

In particular, the Examiner has already accepted the existence of high throughput *in vitro* assays for identifying glycosyltransferase inhibitors,[18] essentially as described in the specification.[19] The only issue raised with respect to such *in vitro* assays is their predictive or correlative value *vis a vis in vivo* efficacy.[20] Accordingly, and in view of the Examiner's previous remarks regarding the delivery of an inhibitor to a target cell *in vitro* or *in vivo*, Applicants believe the Examiner's assertion of lack of guidance (and alleged unpredictability) to be directed toward inhibitor activity in cells, either in *in vitro* cell culture or *in vivo* in a mammal.

In this regard, the specification provides guidance as to desirable characteristics of the inhibitor for use on cells. For example, the specification states the following:

> The preferred glycosyltransferase inhibitors of the present invention have the ability to cross the cell membrane and enter the Golgi apparatus. Thus, the ... agents are preferably sufficiently hydrophobic to allow the diffusion through the membrane.... The ... agents are preferably relatively small molecules, thereby avoiding immunogenecity and allowing passage through the cell membrane. Ideally, they have a molecular weight of between about 100-2000 daltons, but may have molecular weights up to 5000 or more, depending upon the desired application. In most preferred embodiments, the inhibitors have molecular weights of between about 200-600 daltons.[21]

Also, as noted in Applicants' previous Amendments, the specification explains that substrate analogs of glycosyltransferases have been used successfully *in vivo* for other indications, particularly as antibiotic and antibacterial agents. The specification explains, for example, that the replacement of the diphosphate bridge with a carbon chain in tunicamycin, an

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[17] MPEP § 2164.05(a) (citing cases).
[18] *See* Office Action dated February 17, 2005, at p. 3.
[19] *See* specification at pp. 27-31; *see also* Amendment dated April 19, 2005, at p. 5.
[20] *See* Office Action dated February 17, 2005, at p. 3.

analog of UDP-GlcNAc, "allows tunicamycin to cross the cell membrane but still readily bind the active site of the enzyme."[22] The specification further points to such compounds, used successfully *in vivo*, as providing "one of skill in the art with direction in designing and synthesizing compounds with similar inhibitory effects in accordance with the present invention."[23]

The guidance in the specification, as summarized above, is sufficient to allow one of skill in the art to carry out inhibition of C2GlcNAc transferase in cells, either *in vitro* or *in vivo*, in view of the state of the pertinent art. As noted in Applicants' response regarding the state in the art (see above), the only evidence provided by the Examiner as alleged support for a lack of predictability pertains to translocation of larger polypeptides (27 amino acids or more) or antisense oligonucleotides across cellular membranes. None of the cited references are pertinent to the ability of small molecules, such as substrate analogs of glycosyltransferases, to pass through cellular membranes. Moreover, consistent with the specification's teaching regarding inhibitors useful on cells, and as evidenced by Camenisch *et al.* (Exhibit C), it was indeed well-known that small molecules can predictably penetrate cellular membranes by passive diffusion,[24] and that this ability could be readily predicted based on the compound's lipophilicity and molecular size.[25] Still further, as a predictor of bioavailability, the ability of a pharmacologically active compound to penetrate biological membranes would be regarded by the skilled artisan as having predictive value with respect to *in vivo* efficacy. Accordingly, in view of the above in conjunction with the previous *in vivo* use of structurally related compounds, the skilled artisan would regard the specification as providing sufficient guidance for the artisan to carry out C2GlcNAc transferase inhibition *in vivo* using substrate analogs of the enzyme.

In light of the above, Applicants respectfully disagree with the Examiner's assertion that the specification's disclosure of null mice is "not representative of the ability to successfully target and inhibit C2 GlcNAc transferase in vivo comprising the administration of

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[21] Specification at p. 23, ll. 3-9.
[22] *Id.* at p. 21, ll. 26-29.
[23] *Id.* at p. 21, l. 21 to p. 22, l. 2.
[24] *See* Exhibit C.
[25] *See id.* at, *e.g.*, p. 313 (Abstract).

inhibitors." The Examiner has already accepted that the specification indeed teaches an *in vivo* effect of C2GlcNAc transferase inhibition.[26] Further, it appears that the Examiner also accepts that this effect is correlative of an effect that can be achieved if effective inhibitor concentrations can be obtained *in vivo*.[27] For the reasons set forth above, the skilled artisan would readily accept that a substrate analog inibitor C2 GlcNAc transferase, having the appropriate hydrophobicity and molecular size as determined according to routine methods, could be delivered to cells *in vivo* to achieve effective inhibitor concentrations.

The breadth of claims and the quantity of experimentation

Applicants also disagree with the Examiner's contention that undue experimentation would be required to carry out the full scope of the claimed method in view of the quantity of experimentation, including "*de novo* experimentation of accessible target sites, modes of delivery and formulations to target appropriate cells and/or tissues harboring core 2 GlcNAc transferase."[28]

First, with regard to the breadth of the claims, Applicants again note that the present claims now recite substrate analogs as the inhibitory compound. Thus, any bases for this aspect of the rejection pertaining to targeting and delivery of antisense molecules or polypeptides are obviated.

Further, according to the Federal Circuit and the MPEP, the quantity of any experimentation is not determinative:

> The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.[29]

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[26] *See* Office Action dated August 24, 2005, at p. 10.
[27] *See id.* at p. 10, ll. 12-15.
[28] *See* Office Action dated August 24, 2005, at pp. 6 & 7.
[29] *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988); MPEP § 2164.06.

In this case, for reasons already set forth, the specification provides sufficient guidance with respect to the direction in which experimentation should proceed. To briefly reiterate, the specification explains that methods for designing inhibitors based on analogs of the enzyme substrate were well-known and that such inhibitors have been successfully used *in vivo* for other indications.[30] Furthermore, the specification points to inhibitors having the appropriate lipophilicity and molecular size to allow penetration across the cell membrane so as to exert their effects intracellularly.[31] As noted above, it was well-known that the ability of a pharmacologically active compound to penetrate cellular membranes by passive diffusion is an indication of that compound's ability to be absorbed and to reach its site of action.[32]

Thus, based on known methods for assessing physiochemical parameters of candidate molecules, including membrane permeability and lipophilicity,[33] in conjuction with known methods for designing substrate analog inhibitors of glycosyltransferases, the skilled artisan would be able to readily identify inhibitors that can achieve effective intracellular concentrations and, therefore, likely have *in vivo* activity. As the Examiner has already accepted that the specification teaches an *in vivo* effect of C2GlcNAc transferase inhibition, the *in vivo* activity of such inhibitors could be readily confirmed in known animal models, which is standard in the art of drug design. Therefore, in light of the above, and based on the history of *in vivo* use of this particular class of inhibitory compounds, the skilled artisan would reasonably accept that C2GlcNAc transferase inhibitors having the requisite *in vivo* activity could be readily identified, and that any experimentation in carrying out the method as presently claimed would not be undue.

Accordingly, for the reasons set forth above as well as for reasons previously of record, Applicants believe the present claims to be enabled by the specification under 35 U.S.C. § 112, first paragraph. Withdrawal of the rejection is respectfully requested.

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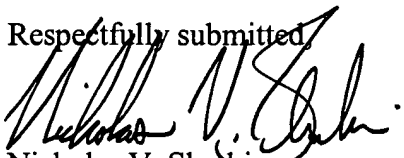
[30] *See* specification at pp. 20-22.
[31] *See id.* at p. 23, ll. 3-12.
[32] *See* Exhibit C at, *e.g.*, p. 313, cols. 1 & 2 (esp. col. 2, first full para., stating that passive diffusion "is the most significant transport mechanism for the majority of drugs").
[33] *See* generally Exhibit C.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 206-467-9600.

Respectfully submitted,



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Estimation of permeability by passive diffusion through Caco-2 cell monolayers using the drugs' lipophilicity and molecular weight

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Abstract

A recently developed, new theoretical absorption model for passive diffusion through biological membranes describing the dependency of membrane permeability on lipophilicity and molecular size, predicts different sigmoid–hyperbolic permeability–lipophilicity relationships for different molecular weight ranges. This model has been tested with experimental in vitro cultured epithelial cell (Caco-2) permeability data for structurally diverse drugs differing in lipophilicity, ionization state and molecular size. These data were pooled with literature values. Using this simple physicochemical approach, the permeability of a compound through Caco-2 cells by passive diffusion can be predicted from the compounds' distribution coefficient in 1-octanol/water (log D_{oct}) and its molecular weight (MW). Deviations from this expected behaviour may point to the involvement of biological components in the transport process, which may require further investigations.

Keywords: Caco-2 cell monolayers; Passive diffusion; Absorption prediction; Lipophilicity; Molecular weight; Membrane permeation

1. Introduction

The in vitro determination of the permeability through cultured cell monolayers has been suggested for the estimation of oral intestinal drug absorption (Artursson, 1991; Hillgren et al., 1995; Conradi et al., 1996). To this end, the human intestinal cell line Caco-2 is an often-used in vitro model for epithelial drug uptake, since these cells express various biological membrane properties, including active transport systems and tight junctions.

For large series of compounds, e.g. from combinatorial chemistry projects, the measurement of permeation through cell cultures becomes rapidly unpractical with todays technologies. Therefore, there is currently great interest in evaluating the role of physicochemical properties in membrane permeation processes, such as gastrointestinal absorption and BBB uptake (Chan and Stewart, 1996; Artursson et al., 1996; Burton et al., 1996; Palm et al., 1996, 1997; Van de Waterbeemd, 1997; Van de Waterbeemd et al., submitted). The background of present studies is to develop high-throughput systems for permeability/absorption estimation.

A biological membrane, consisting of an aqueous (paracellular) and lipoidal (transcellular) pathway in parallel, can be characterized by its lipophilic character, the presence of water pores and the existence of stagnant diffusion layers. Passive diffusion is the most significant transport mechanism for the majority of drugs, with the physicochemical properties of both the drug and the permeation barrier being the major rate determinants for transport. As recently reviewed, passive diffusion depends to a large extent on three interdependent physicochemical properties, namely lipophilicity (log P or log D), polarity (charge, hydrogen bonding) and molecular size (Camenisch et al., 1996). Lead optimization for oral delivery involves the multidimensional optimization of these properties.

Charge has been studied as a factor of putative importance for membrane transfer (Adson et al., 1994; Rubas et al., 1994; Pauletti et al., 1996; Tamura et al., 1996; He et al., 1996). However, its role its controversial. In a recent study on oral absorption of oligopeptides in the rat, it was concluded that net charge showed little effect on bioavail-

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ability (He et al., 1996). In our view, charge mainly contributes to the attraction or repulsion of a drug by the membrane surface and/or tight junctions. For the membrane transport itself, charge is accounted for in the distribution coefficient log D. Further studies should address whether a distinction has to be made for the charge effect on paracellular versus transcellular permeation.

Lipophilicity has two principal components, namely molecular size and hydrogen bonding (Testa and Seiler, 1981; El Tayar et al., 1992). The role of hydrogen bonding in membrane permeation had recently gained much attention (Conradi et al., 1996; Van de Waterbeemd et al., 1996; Palm et al., 1996, 1997), but will not be further discussed in the present contribution.

Molecular size has been shown to be not only a component of lipophilicity (Testa and Seiler, 1981; El Tayar et al., 1992), but also of the diffusion coefficient D in biological membranes and continuous fluid media (Lieb and Stein, 1971; Walter and Gutknecht, 1986; Xiang and Anderson, 1994). For diffusion in water this dependence is relatively small, while for transcellular diffusion in biological membranes, a rather strong dependence on molecular size was observed. The paracellular diffusion through the tight junctions of a biological membrane is, because of the restricted size of these aqueous pores, also dependent upon molecular size (Leahy et al., 1989). In summary, three different size effects on membrane permeation may be defined, namely diffusion, sieving and distribution effects. As a simple molecular size descriptor often the molecular weight MW is used (Chan and Stewart, 1996; Camenisch et al., 1996; Van de Waterbeemd et al., 1996; Leahy et al., 1989). However, for larger compounds, such as peptides, it may be better to define a hydrodynamic size or radius (Adson et al., 1994). Shape aspects of membrane transport are beyond the scope of the present paper (Van de Waterbeemd et al., submitted).

Recently we developed a theoretical model (extended two-step distribution model), describing the passive transport through a biological membrane as a function of the permeation pathway and the molecular physicochemical properties lipophilicity and size (Camenisch 1996; Camenisch et al., submitted). Briefly, we expect a sigmoidal relationship between permeability and lipophilicity for compounds within a narrow MW range. This implies that in fact a set of sigmoidal curves should be observed. For higher MW, these curves should shift to lower permeabilities. A similar permeability–lipophilicity–molecular weight relationship model has been proposed by others (Leahy et al., 1989). In the present study, this model is tested using experimental permeability data from Caco-2 cell monolayer studies using a set of structurally diverse compounds. The compounds were selected to cover a broad range in molecular weight and lipophilicity. We also address the question of pooling Caco-2 permeability data from different laboratories. At the time of selection of the compounds, most were expected to be absorbed exclusively or mainly by passive diffusion (Artursson and Karlsson, 1991; Karlsson et al., 1993). More recent studies have identified a number of the selected compounds as a ligand for P-glycoprotein (P-gp) efflux or as being transported by active carriers (Wacher et al., 1996). Furthermore the absorption of certain compounds may be limited by intestinal phase I, particularly CYP3A4, metabolism (Wacher et al., 1996). However, usually the extent of these processes is not known. Our results demonstrate that despite these potential limitations, well-defined relationships between Caco-2 cell permeability and physicochemical properties have been obtained, thereby confirming the theoretically expected trends.

2. Experimental

2.1. Selected compounds

The following compounds have been considered (n= 35): Alprenolol(HCl), atenolol, bosentan, ceftriaxone(Na), corticosterone, coumarin, dexamethasone, [^{3}H-]diltiazem, [^{3}H-] epinephrine, felodipine, fleroxacin, furosemide, guanabenz, guanoxan, hydrocortisone, [^{3}H(N)-]imipramine(HCl), [^{14}C-]lidocaine(HCl), [^{3}H-]mannitol, metoprolol($C_4H_6O_6$), [^{3}H-]nitrendipine, olsalazine(Na), mibefradil(HCl), practolol, propranolol(HCl), proscillaridin, [^{3}H-]remikiren, saquinavir, sulphasalazine, [^{3}H-]sulpiride, terbutaline($1/2H_2SO_4$), theophylline, tiacrilast, [^{14}C-]testosterone, (±)verapamil(HCl) and [^{14}C-]warfarin. The molecules cover a relevant range of molecular weight from 140 up to 671 g/mol and a relevant wide range of lipophilicity (Table 1). The cold (not radiolabeled) drugs all show UV-absorption.

All substances and tissue culture reagents were ordered from Sigma Chemie (Switzerland). Olsalazine (Pharmacia AB, Sweden), testosterone (Roche Bioscience, USA), felodipine (Astra Hässle AB, Sweden), practolol (Zeneca Pharmaceuticals, UK) and guanoxan (Pfizer Ltd., UK) were generous gifts. Bosetan, fleroxacin, mibefradil, ceftriaxone and saquinavir were obtained in-house (F. Hoffmann–La Roche, Switzerland).

2.2. Caco-2 cell cultures

Caco-2 cells with a passage number of 100–110 were used. Cells were kept frozen in aliquots in liquid nitrogen. After thawing, the cells were routinely maintained in flasks in Dulbeccos's Modified Eagle Medium (pH7.4), containing 10% calf serum, 1% nonessential amino acids, 1% L-glutamine and penicillin (100 U/ml)/streptomycin (100 μg/ml) in an atmosphere of 5% CO_2 at 90% relative humidity. Cells grown in 80 cm^2 tissue culture flasks were passaged every week by trypsinization. After a maximum of 10 passages, cell culture was started again with passage

Table 1
Physicochemical properties and absorption characteristics of the drugs

Compound	MW	log P^a	log D^a_{oct}	log P^c_{erm}
Coumarin	146	1.39	1.39	−4.11
Theophylline	180	−0.02	−0.02	−4.35
Mannitol	182	−3.10	−3.10[d]	−5.58/−6.74[d]
Epinephrine	183	−1.75	−2.59[e]	−6.02
Guanoxan	194		−0.83[b]	−4.71
Terbutaline	225		−1.40[d]	−6.42[d]
Guanabenz	231		1.67[b]	−4.14
Lidocaine	234	2.26	1.63	−4.21
Alprenolol	249	3.10	1.00[d]	−4.39[d]
Propranolol	259		1.54[d]	−4.38[d]
Tiacrilast	262		−1.05[b]	−4.90
Practolol	266	0.79	−1.40[d]	−5.86/−6.05[d]
Atenolol	266	0.16	−2.14[d]	−6.63/−6.70[d]
Metoprolol	267	1.88	0.07[d]	−4.57[d]
Imipramine	280	4.80	2.52	−4.26
Testosterone	288	3.32	3.31[d]	−4.23/−4.29[d]
Olsalazine	302		−4.50[d]	−6.96[d]
Warfarin	308	2.70	0.12[d]	−4.27/−4.42[d]
Furosemide	331	2.03	−1.21[b]	−6.09
Sulpiride	342		−1.15	−6.16
Corticosterone	346	1.94	1.89[d]	−4.26[d]
Nitrendipine	360		0.97	−4.77
Hydrocortisone	362	1.61	1.53[d]	−4.67[d]
Fleroxacin	369	−0.55	−0.29[b]	−4.81
Felodipine	384		3.48[d]	−4.64[d]
Dexamethasone	392	1.83	1.74[d]	−4.90[d]
Sulphasalazine	398		−0.39[b]	−6.89[d]
Diltiazem	415	2.80	2.22[e]	−4.31
Verapamil	455	3.79	2.07[b]	−4.58
Mibefradil	496		3.66[b]	−4.87
Bosentan	522		1.30[b]	−5.98
Proscillaridin	531	2.48	2.48	−6.20
Ceftriaxone	555		−1.23[b]	−6.88
Remikiren	631		2.75[b]	−6.13
Saquinavir	671		3.00[b]	−6.26

[a] 1-Octanol/water distribution coefficient at pH 7.4 from the MedChem95 database.
[b] Experimental 1-octanol/water distribution coefficient at pH 7.4 from current study.
[c] Experimental Caco-2 permeability data (s.d. <5%) from current study.
[d] Data from Artursson and Karlsson (1991).
[e] Calculated from log P and pK_a.

number 100. For transport studies, 70 000 Caco-2 cells were seeded per Transwell™ microporous cell culture insert (pores: 0.4 μm, area: 1.13 cm^2). The inserts were placed in wells in normal cell culture plates and were allowed to grow for at least 21 days. The medium was changed every 2–3 days. The confluent monolayers were used between days 21 and 28 after seeding. All cell culture equipments were purchased from Costar.

2.3. Transport studies

Transport experiments were initiated by transferring the cell culture inserts to a Costar 12-well plate. A mixture of 100 ml Hanks' balanced salt solution (HBSS, 10x), 11 ml HEPES buffer solution (1 *M*), 20 m*M* glucose monohydrate, 5 m*M* sodium hydrogen carbonate and water 1000 ml at pH 7.4 was used as transport buffer (pH 7.4). Thus, both donor and acceptor chamber are of same pH. Drug samples (1 m*M* or saturated solution <1 m*M* in the case of highly lipophilic compounds such as testosterone) were added to the apical chamber (0.5 ml), and transport buffer without drug was added to the basolateral chamber (1.5 ml). The integrity of the monolayer was checked by the addition of 0.01 mg/0.5 ml Lucifer yellow (LY) to the apical side of the monolayer and determination of the amount of LY appearing in the basolateral compartment. The critical maximum flux of LY to identify leaky monolayers was estimated to be <1% of starting concentration. Samples of 100 ml were transferred into MICROFLUOR™ 96-well plates (Dynatech) and fluorescence (excitation 420 nm, emission 515 nm) was determined with a Perkin Elmer luminescence spectrophotometer LS 50B and a Perkin Elmer plate reader. Transport rates were determined by replacing the medium in the basolateral chamber after 15, 45, 135 and 360 min (corresponding to intervals of 15, 30, 90 and 225 min). This was accomplished by moving the insert itself to a new well containing fresh medium at each time point. Solutions were not stirred or agitated during transport experiments.

Each compound was studied in triplicate. The drug concentrations in starting samples, in collected samples in the acceptor compartment and drug remaining in the donor compartment at the end of the experiment were determined. The mass balance was checked and was better than 90%. The amount of radiolabeled drug was quantitated by liquid scintillation counting in a Beckmann LS-5801 apparatus. Cold drug was quantitated by UV-absorption measurements in a SPECTRAmax 250 microplate spectrophotometer. For all samples taken at the time point 360 min, a RP–HPLC (Supelco ABZ-column with water–methanol gradient over 30 min) chromatogram was recorded for inspection of additional peaks, in order to verify that no cell particles, being photometrically active and therefore disturbing quantification, are liberated into the basolateral side, and/or metabolites have been formed.

2.4. Data analysis

Permeability coefficients through the Caco-2 cell monolayer were calculated from the following equation (Hilgers et al., 1990; Camenisch et al., 1997):

$$P_e = \frac{V_A}{A \cdot (C_D - C_A)} \cdot \frac{dC_A}{dt} \quad (1)$$

where dC_A/dt (mg/s/ml) is the increase of drug concentration in the acceptor chamber during a time considered, A (=1.13 cm^2) the surface area exposed to the compound, V_A the solvent volume in the acceptor chamber

(ml) and C_D the initial concentration of solute in the donor chamber (mg/ml).

P_e values calculated from Eq. (1) are only correct under experimental conditions that provide constant concentration gradient, i.e. that C_D is almost constant and C_A is negligible compared to C_D. In the literature, experimental conditions are usually accepted as sufficiently accurate for P_e calculations, if the concentration difference between donor and acceptor compartment does not diverge by more than 10% in the time interval studied (Anderberg et al., 1991). The sampling intervals in this study (15, 30, 90 and 225 min) were selected to study the transport of drugs with a broad range of physicochemical properties and transport characteristics (paracellular versus transcellular diffusion) under the same experimental conditions. Since we are developing a medium-throughput system, sampling intervals were not individually adjusted. Therefore, sampling intervals were not always optimal for rapidly transported compounds to achieve sufficient sink conditions. In these cases only the highest P_e value (usually at the 15-min interval) was used (warfarin, coumarin, theophillin, diltiazem, lidocain, imipramine, testosterone, demeclocycline). In all other cases P_e values were calculated for each time interval and the average P_e value was used.

Data analysis was performed using Statistica 5.0 for Windows (StatSoft Inc., USA).

2.5. Experimental data

Caco-2 experiments were designed based on the work of Artursson's group (Artursson and Karlsson, 1991), since it was our intention to combine our data with previously measured compounds in order to have a larger data set. Preliminary Caco-2 cell permeation studies were performed using atenolol, practolol, warfarin, testosterone, and mannitol. This subset of compounds spans a lipophilicity (log D) range of -3.1 (mannitol) to $+3.3$ (testosterone). With the exception of mannitol, it was demonstrated that our permeability data are well correlated with (Fig. 1) and quantitatively nearly identical to data in the literature (Artursson and Karlsson, 1991). Therefore, we pooled our present new data with those for alprenolol, propranolol, terbutaline, felodipine, metoprolol, corticosterone, hydrocortisone, olsalazine, sulphasalazine and dexamethasone reported previously. The hydrophilic compound mannitol has a MW$<$200 and is known to permeate via the paracellular aqueous pore pathway (Artursson et al., 1996). Since Caco-2 cells are a heterogenous cell population, interlaboratory differences have been demonstrated (Artursson et al., 1996). As our measurements and those of others show that particularly, the extent of permeation via the paracellular pathway seems to vary. Most of the compounds, i.e. all compounds with MW$>$200, in the present study are believed to use the transcellular route.

Experimental 1-octanol/water distribution coefficients (log D) at pH 7.4 were taken from the MedChem95 database (Daylight Chemical Information Systems, USA) or determined using the shake flask or pH-metric method (Avdeef, 1996). All experimental data are presented in Table 1.

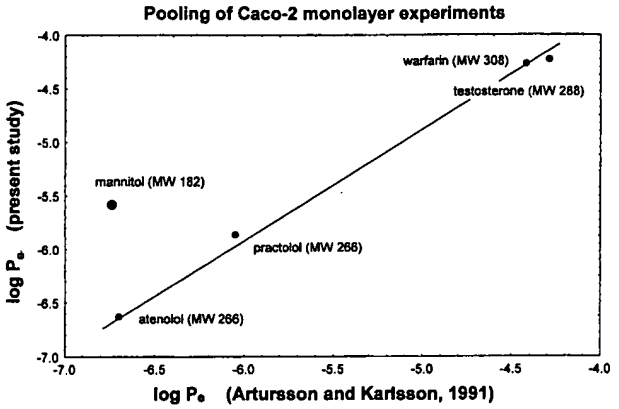

Fig. 1. Comparison of permeability in Caco-2 monolayers in two different laboratories, but using the same experimental conditions. Only mannitol, transported uniquely via the paracellular route, shows interlaboratory variations.

2.6. Permeability–lipophilicity relationships

Transport through a membrane can be described by the permeability coefficient P_e (cm/s), which is put together as follows:

$$P_e = P_e^{lip} \cdot f^{lip} + P_e^{hyd} \cdot f^{hyd} \quad (2)$$

where the superscript hyd denotes the aqueous part (pores, paracellular route) and the superscript lip the lipoidal part (transcellular route) of the membrane and where f^{lip} and f^{hyd} are the fractional areas of these parallel routes.

Based on Eq. (2), we derived a sigmoidal permeability–lipophilicity relationship described by Eq. (3) (Camenisch, 1996; Camenisch et al., submitted). Preassumptions in this model are stagnant aqueous diffusion layers on both sides of the monolayer, identical pH in the aqueous donor and acceptor compartments, and the compounds should have comparable MW.

$$\log P_e = a \cdot \log(1 + \alpha \cdot D_{oct}) - a \cdot \log(1 + \beta \cdot D_{oct}) + b \quad (3)$$

In this equation, D_{oct} is the measured 1-octanol-water distribution coefficient. α, β, a and b are parameters to be estimated by nonlinear regression. Similar to earlier studies (Stehle and Higuchi, 1972) the tailing effect at high lipophilicity values is believed to be due to stagnant aqueous diffusion layers in front of the membrane. The tailing effect at low lipophilicity values is a function of paracellular diffusion. Thus, for compounds unable to permeate via the paracellular pathway, the sigmoidal relationship simplifies to a simple hyperbolic or bilinear relationship according to:

$$\log P_e = a \cdot \log D_{oct} - a \cdot \log(1 + \beta \cdot D_{oct}) + b \quad (4)$$

Since paracellular diffusion shows a molecular size dependence due to the sieving effect and transcellular diffusion is size-dependent due to a diffusion effect, different curves are expected for different molecular weight ranges.

2.7. The influence of molecular weight

Compounds with MW<200 are able to pass the intestinal mucosa by the paracellular pathway (Lennernäs, 1995). Nevertheless, in parallel, part of the transport may occur via the transcellular route (Chan and Stewart, 1996). Inspection of the World Drug Index (WDI) reveals that most common drugs have a MW in the range 300–400 (Rose et al., 1994). Tentatively, three molecular weight ranges can be defined, each range representing a zone of different molecular size effects. For small compounds with MW below 200 pore diffusion may occur ('nonrestricted or restricted pore diffusion'). For compounds with a MW around 200, a sieving effect becomes perceptible. Compounds with a molecular weight of about 350±150 can readily diffuse through the membrane without any restriction. This is the range of 'nonrestricted membrane diffusion'. Increasing the MW further (approximately MW> 500) leads to a decrease in membrane diffusion thus defining the MW range of 'restricted membrane diffusion'. An alternative for high MW compounds is to permeate the membrane via endocytosis/transcytosis (Artursson et al., 1996).

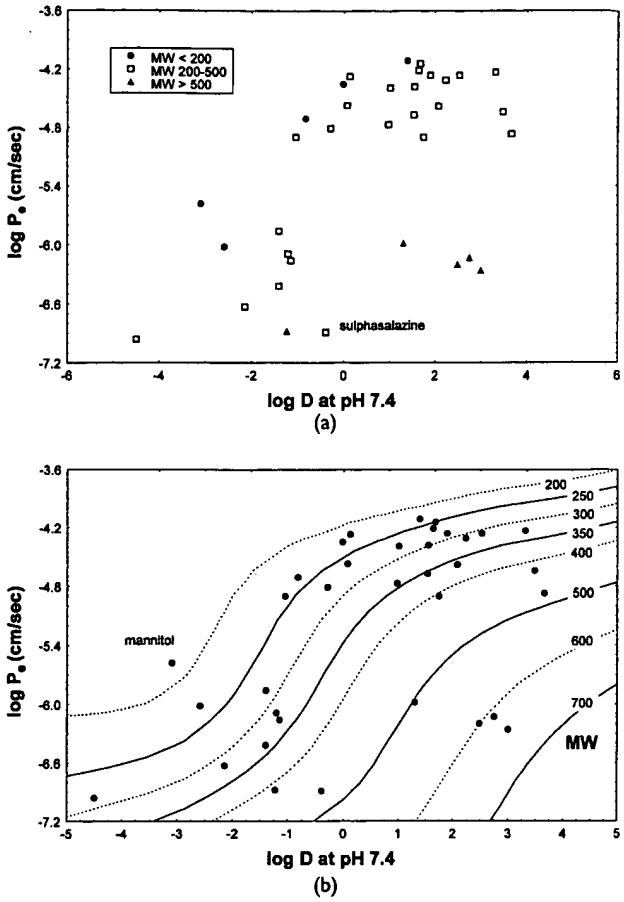

Fig. 2. Influence of the drugs' distribution coefficient measured in 1-octanol/water (log D) and the effect of molecular weight (MW) on permeability through Caco-2 monolayers: (a) using different MW ranges, (b) using MW contour plots (using a spline function for curve fitting).

3. Results

The Caco-2 cell permeability data (log P_e) are plotted in Fig. 2 against experimental 1-octanol/water distribution coefficients (log D at pH 7.4). In Fig. 2a three MW ranges have been marked by different symbols. It is observed that the smallest compounds (MW<200) are on the left side on the plot, while the larger ones (MW>500) are found on the right side. The majority of compounds with a MW 200–500 falls somewhere in between these extremes. Sulphasalzine forms an exception, being more closer to the high MW compounds. No rational can be offered for this behaviour. However, it should be realized that experimental errors, both in log D and log P_e have an impact on the exact position of each point. The purpose of the present paper was to uncover trends in the influence of MW on permeability. Therefore in Fig. 2b, contours are presented using the molecular weight as third descriptor. The data are fitted using the spline-smoothing procedure. From this contour plot is seen, that for the different molecular weight ranges, separate curves can be drawn. For the low and medium molecular weight (MW up to ca. 500) compounds well-defined sigmoidal relationships are observed, as predicted by our model (Camenisch, 1996; Camenisch et al., submitted and Eq. (3) and as proposed by others (Leahy et al., 1989). The higher MW ranges (MW>500) are not so well-defined due to the limited number of experimental data. However, with increasing molecular size the sigmoidal curves seem to change into more or less simple hyperbolic (also called bilinear) functions, as described by Eq. (4). This is compatible with the fact that compounds having higher lipophilicities and higher molecular weight cross the epithelium predominantly via the transcellular route. Future studies of permeabilities through Caco-2 monolayers should particularly cover this high MW range in order to obtain better defined curves.

4. Discussion

An important result of the present study is that the position of a permeability–lipophilicity curve on the lipophilicity axis should be considered as a function of MW. Therefore the steep part of the curve, i.e. the critical range for oral absorption, may be at different lipophilicity

values, according to the MW of the series of compounds under study.

The effect of increasing the lipophilicity, defined here as the distribution coefficient in 1-octanol/water, on permeability is clearly demonstrated in Fig. 2. For a particular MW range, e.g. 300–400, we propose to define several lipophilicity ranges, each range representing a zone of different distribution behaviour. The lowest range comprises log D_{oct} values where compounds are too hydrophilic for any distribution into the membrane. Above a threshold, transcellular membrane diffusion becomes possible and describes the lower curvature of the sigmoid. At ca. log $D_{oct} = -1$ to 0 the inflection point of the sigmoidal curve is found and a steep dependence of permeability on lipophilicity is observed. This may be critical in the lead optimization process. Minor structural modifications may increase the log D only slightly, but have a large effect on permeability and absorption. The next lipophilicity range describes the upper curvature of the sigmoid. Above log $D_{oct} = 2$ compounds permeate readily and lipophilicity-independent through Caco-2 cells. Finally, increasing the lipophilicity too much, leads to solubility-limited absorption.

According to current experimental data, the molecular weight cut-off for the Caco-2 cell permeability is at MW ca. 700 (Fig. 2), slightly higher then the MW 600 estimated by others (Artursson et al., 1993). However, the largest studied compounds here are renin inhibitor remikiren (MW 631) and HIV inhibitor saquinavir (MW 671), both peptidic compounds. Further studies should reveal whether peptides of MW > 600 behave differently or fit under certain conditions into the current model.

The physicochemical graphical model presented here allows us now the ab initio prediction of passive diffusion through Caco-2 cells, and thus indirectly of human intestinal absorption (Artursson and Karlsson, 1991). Prerequisite is the experimental determination of appropriate distribution coefficients. As demonstrated, the 1-octanol/water solvent system fulfils this role. To our opinion this is because this solvent mimics quite well the hydrogen bonding properties of epithelial membranes. This approach can be combined with the evaluation of H-bonding potential (Conradi et al., 1996; Burton et al., 1996; Van de Waterbeemd et al., submitted; Van de Waterbeemd et al., 1996; Palm et al., 1996, Palm et al., 1997). Present discussion is limited to low MW drugs. Peptides may require additional studies (Burton et al., 1996).

It can be speculated that active or efflux-hindered transport of compounds is contributing to the permeation process, if higher and respectively lower permeabilities are measured than predicted from their physicochemical properties. Caco-2 cells may thus be used to confirm predictions based on the present model for a selected set of compounds, e.g. representatives of a structurally new series of compounds. Furthermore, in vitro cell cultures may be of interest to study intestinal metabolism (Wacher et al., 1996).

It should now be possible to derive similar physicochemical absorption models for other biological barriers, such as the intestinal tract, blood–brain barrier or even the skin. Differences between these diffusion barriers are found in the size of the aqueous pores and the composition of the lipoidal membrane. Probably, this will shift the curves presented here for Caco-2 cells to different positions. However, the fundamental biophysical processes of transfer through any of these biological barriers should be very similar (Burton et al., 1996). As an example we have recently demonstrated that this approach can also be used to predict permeability through the rat intestine, using data from a single-pass perfusion model (Van de Waterbeemd, 1997).

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